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Enzyme-luminescence method: Tool for real-time monitoring of natural neurotoxins in vitro and L-glutamate release from primary cortical neurons

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ABSTRACT

Novel enzyme-luminescence method is used for the rapid and sensitive in vitro detection of natural neurotoxins (e.g., shellfish and mushroom toxins) using model brain cells. Paralytic shellfish poisons gonyautoxins (e.g., GTX2,3 and GTX1,4) were detected at 1 nM level by their inhibition of glutamate release from C6 glioma cells upon drug stimulation (IC_{50}: GTX2,3 = 30 nM and GTX1,4 = 8 nM). Activation of glutamate release from C6 cells by ibotenic acid (a mushroom toxin) was also evaluated (EC_{50} = 10 nM). The method was tested for real-time detection of glutamate release from primary rat cortical neurons. Dose-dependent effects of KCl (0–200 mM) and NMDA on glutamate release from primary cortical neurons were studied. The effects of different culture conditions on K^+-depolarization-induced glutamate release were also investigated. The method may be applicable to screening of drugs and toxins, and finding glutamatergic neurons in brain slices without in situ staining.

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1. Introduction

Marine poisons (e.g., paralytic shellfish poisons) and mushroom toxins (e.g., ibotenic acid) are extremely neurotoxic compounds due to their potent toxicity to the nervous systems of humans. A number of paralytic shellfish poisons (PSPs) are known to be responsible for the inhibition of glutamate release from cells. Ibotenic acid, isolated from Amanita muscaria and Amanita pantherina also stimulates glutamatergic neurons or glial cells through *N*-methyl-D-aspartic acid (NMDA) receptor-mediated elevation of intracellular Ca^{2+}, which can lead to exocytosis of L-glutamate release from cells [1–3]. Glutamate, a principal excitatory neurotransmitter [4,5–7], is responsible for several neurological disorders, including strokes, epilepsy, schizophrenia, Alzheimer's disease, and Parkinson's disease [8,9]. Therefore, the natural toxins such as marine poisons and mushroom toxins can be detected indirectly by in vitro monitoring of extracellular L-glutamate release. The chemical structures of gonyautoxins (e.g., GTX1, GTX2, GTX3, GTX4) and ibotenic acid (a mushroom toxin) are shown in Scheme 1.

Voltage-dependent Na^+ channel is critical for depolarization and conduction in most excitable cells. Paralytic shellfish toxins, a family of potent neurotoxins block the voltage-gated Na^+ channel (VGSC). A schematic illustration of the induction mechanism of the activator (e.g., veratridine (VTRD) which activates voltage-dependent Na^+ channels) and the inhibition mechanism of shellfish toxins (e.g., GTX2,3, GTX1,4 which block $Na_v1.4$) of L-glutamate release from a glutamatergic nerve cell or a glial cell is depicted by numbers in squares in Scheme 2. The activation of the NMDA receptor is also important in the release of neurotransmitters, such as L-glutamate [1–3,10–12]. A schematic representation of the action mechanisms of the NMDA receptor agonists (e.g., ibotenic acid and NMDA) is depicted by numbers in circles in Scheme 2. NMDA and ibotenic acid are specific agonists for the NMDA receptors and they help to increase intracellular concentration of Ca^{2+} ions as well as the release of L-glutamate from cells. However, only a very few studies have been conducted on the effects of ibotenic acid on NMDA receptors on the release of glutamate [1–3]. The effects of NMDA are normally determined using fluorescence microscopy with a calcium indicator [10]. However, this technique is labor intensive as the indicator reagent should be loaded into the cell bodies, and any excess of the reagent should be removed by washing each time.

Several analytical techniques have been proposed to detect natural neurotoxins, such as HPLC with pre- and post-column derivatization [13,14], thin-layer chromatography (TLC) [15], fluorometry [16], cell bioassay [17], flow cytometry [18],

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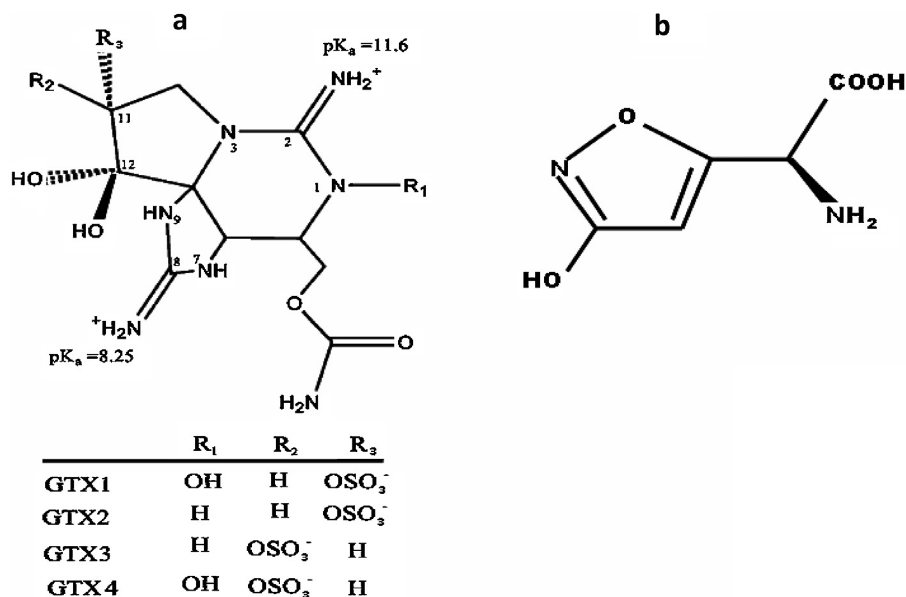
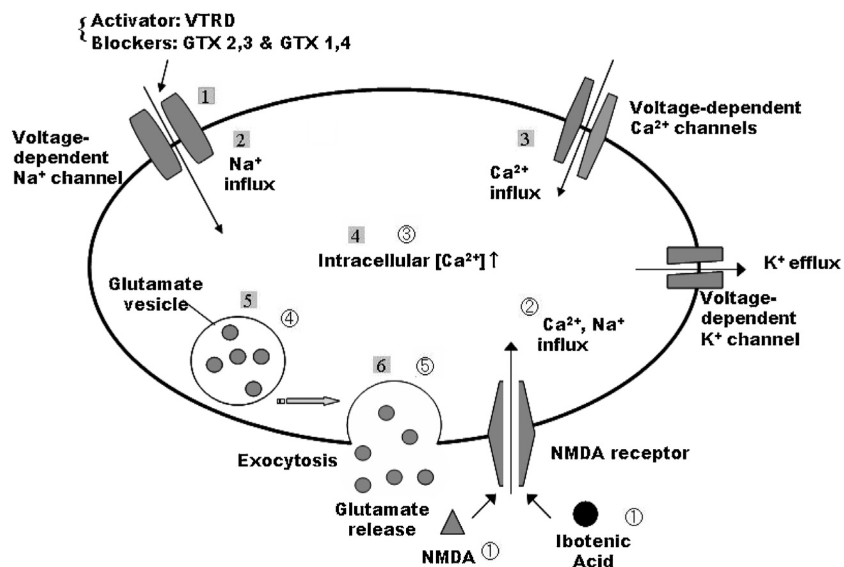


Scheme 1. Chemical structures of (a) paralytic shellfish poisons (e.g., GTX1, GTX2, GTX3, GTX4), and (b) ibotenic acid.

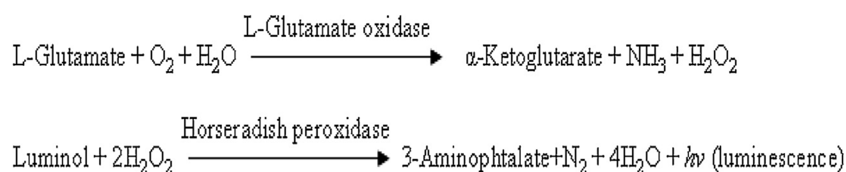


Scheme 2. Schematic representation of mechanisms of the release of L-glutamate from C6 cells by gonyautoxins, a Na^+ channel activator, and NMDA receptor agonists. Veratridine activates voltage-dependent Na^+ channels while ibotenic acid and NMDA activate the NMDA receptor, thereby helping trigger exocytosis of neurotransmitters, such as L-glutamate from the cells through membrane depolarization and the increase of the intracellular Ca^{2+} concentration. Shellfish toxins (e.g., GTX2,3 and GTX1,4) block voltage-dependent Na^+ channels inhibiting the release of L-glutamate.

neurophysiological methods [19], capillary electrophoresis [20,21], hemolysis [22], electrochemical methods [23], electrophoretic method [24], and ELISA [25,26]. An ELISA test kit for detecting saxitoxin in shellfish is also commercially available (RIDASCREEN® Saxitoxin and RIDASCREEN®FAST Saxitoxin; r-Biopharm AG). The pros and cons of these methods is outlined in Table 1. Although all

Table 1
Pros and cons of conventional and enzyme luminescence methods.

Method of analysis	Pros and cons
1. Conventional techniques: HPLC, thin-layer chromatography (TLC), fluorometry, cell bioassay, flow cytometry, neurophysiological methods, capillary electrophoresis, hemolysis, Elisa, electrochemical methods, and electrophoretic method	Not simple and easy to operate, lower sample throughput, labor intensive nature, highly selective and sensitive, real-time monitoring is limited due to the long time required for analysis, staining of the cells with dye is required for some methods, relatively poor spatial resolution
2. Enzyme-luminescence technique	Simple and easy to use, highly selective and sensitive, high sample throughput, good for real-time detection, lower labor costs, applicable for screening of drugs and toxins, has potential in finding glutamatergic neurons without immunostaining, may be suitable to investigate the effects of various growth factors and chemicals on neuronal differentiation, neurotransmitter dynamics, neurodegeneration, and synaptogenesis



Scheme 3. Enzyme-luminol reactions for L-glutamate detection.

of these systems have high selectivity and adequate sensitivity for natural neurotoxins, there still remains a strong interest in finding alternative methods for the detection of these toxins more rapidly and sensitively.

Shinohara and co-workers including the author have proposed a novel enzyme-luminescence technique to detect L-glutamate sensitively and rapidly based on model brain cells (C6 glioma cells) [27,28–30]. The principle of enzyme-luminescence technique, in which L-glutamate is oxidized with glutamate oxidase to produce H_2O_2 that reacts with luminol to generate chemiluminescence in the presence of horseradish peroxidase, is depicted in Scheme 3.

In the first part of this study, the applicability of this system for in vitro model cell-based detection of natural neurotoxins (e.g., paralytic shellfish poisons and mushroom toxin) are demonstrated. In this procedure, racemic mixtures of the marine toxins GTX2,3 and GTX1,4 were administered to C6 glioma cells separately to evaluate the inhibition effects of these toxins on the release of glutamate from the cells upon stimulation by VTRD. Ibotenic acid, a NMDA receptor agonist was administered to C6 cells to induce the release of glutamate. The potency of shellfish poisons and the mushroom toxin was compared by calculating IC_{50} and EC_{50} values, respectively. In the second part of the study, the method is used for the real-time detection of glutamate release from primary rat cortical neurons instead of model cells. Dose-dependent excitatory effects of KCl and NMDA on the glutamate release are also presented. In addition, the effects of different culture conditions on the KCl-induced glutamate release are investigated.

2. Experimental

2.1. Reagents and chemicals

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), KCl, glucose, kanamycin sulfate and sodium glutamate monohydrate were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). L-Glutamate oxidase from *Streptomyces* sp. X-119-6 was obtained from Yamasa Corporation (Choshi, Japan). Peroxidase (POD) from horseradish was purchased from Toyobo Co., Ltd. (Osaka, Japan). Fetal calf serum was obtained from ICN Biomedical, Inc. Ham's F10 medium powder, horse serum, DMEM, and penicillin–streptomycin were obtained from GIBCO. Racemic mixtures of the gonyautoxins (GTX), including GTX2,3 and GTX1,4 were received as a gift from Kitasato University, Japan. α-Amino-3-hydroxy-5-isoxazoleacetic acid (ibotenic acid) from mushrooms, modified Hank's balanced salt (HBSS) powder,

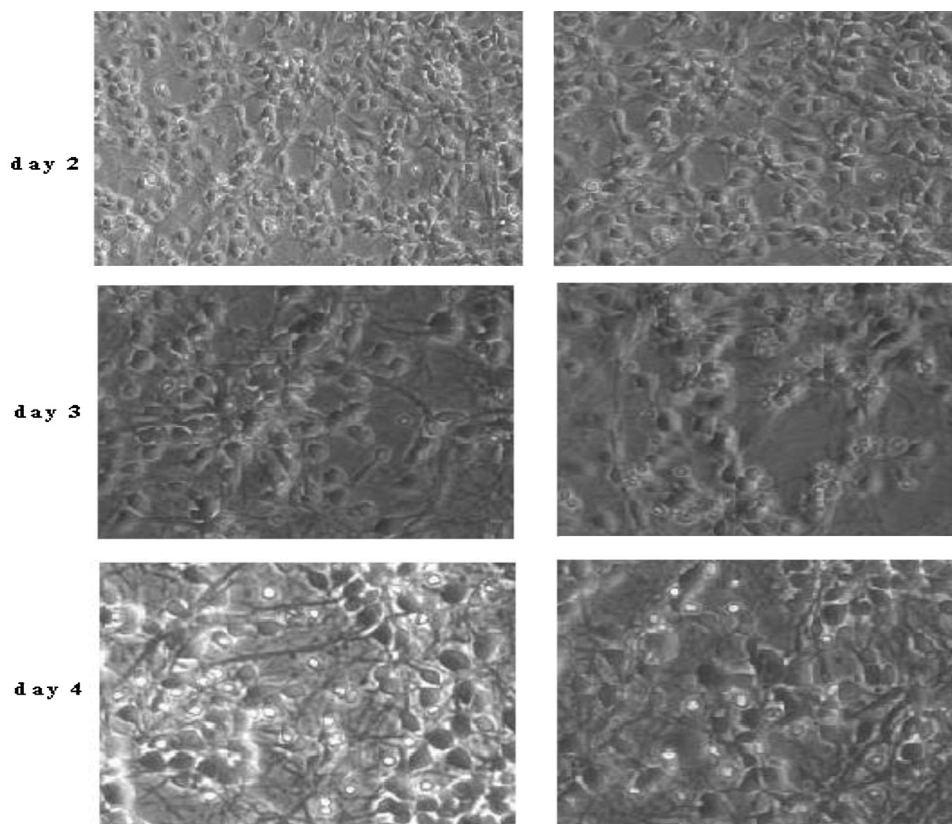
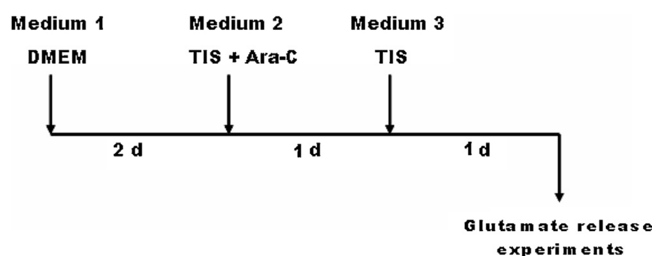


Fig. 1. Phase contrast microscopic images of cultured rat cortical neurons at day 2, 3, and 4. Three different culture conditions, (i.e., DMEM, TIS with Ara-c, and TIS) were used sequentially as depicted in Scheme 3. Primary cortical neurons are differentiated into glutamatergic neurons.



Scheme 4. Different media were used during culturing of primary cortical neurons to differentiate them into glutamatergic neurons.

veratridine (VTRD), insulin-transferrin-sodium selenite (TIS), cytosine arabinoside (Ara-C), albumin from bovine serum, DNase I, trypsin inhibitor and *N*-methyl-d-aspartic acid (NMDA) were purchased from Sigma.

2.2. C6 Glioma cell culture

Rat model cells (C6 glioma cells- JCRB9096 and passage number 45) were obtained from Health Science Research Resources Bank, Osaka, Japan. Cells were grown in a T-25 flask with Ham's F10 medium supplemented with 15% (v/v) horse serum, 2.5% (v/v) fetal calf serum and 1% penicillin–streptomycin at 37 °C with 5% CO_2 in air. Passages of the cells were performed every fourth day using 0.25% trypsin—EDTA for detaching the cells for the duration of cell growth. 24 h prior to the cell assay, 4-day-old cells were inoculated at a density of 1×10^6 cells/mL into a 24-well plate.

2.3. Primary cultures of cortical neurons and differentiation

Primary cultures of cortical neurons were prepared from Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan) at embryonic day 17 or 18 (E17 or E18). In brief, small pieces of cerebral cortex were treated with 0.125% trypsin (DIFCO) and 1 mM EDTA for 15 min at 37 °C and were thoroughly dissociated in DMEM. After centrifugation, the cell pellet was treated with 0.004% DNase I and 0.03% trypsin inhibitor, and the suspended cells were seeded at 1 × 106 cells/mL in a poly-L-lysine-coated 24-well plate. The cells were grown for 2 days in DMEM (supplemented with 10% fetal calf serum and 100 mg/mL kanamycin sulfate) in a 10% CO_2 incubator. The medium was then replaced with serum-free of DMEM supplemented with glucose (4.5 mg/mL), transferrin (5 μg/mL), insulin (5 μg/mL), sodium selenite (5 ng/mL), bovin serum albumin (1 mg/mL), and kanamycin sulfate (100 μg/mL) (TIS medium). Cytosine arabinoside (Ara-C) (final conc. 2 μM) was also added into TIS medium to prevent the proliferation of glial cells. After 1 d of incubation with the TIS medium, the medium was replaced again with fresh TIS medium but devoid of Ara-C. The morphology of cultured neurons is shown in Fig. 1. The L-glutamate release from primary cortical neurons was monitored by evoking KCl or NMDA using an enzyme luminescence method. A scheme depicting the use of different media during culturing is shown in Scheme 4. The rate of differentiation from primary neurons into glutamatergic neurons is thought to be high when three media are used sequentially.

2.4. Monitoring of assay of natural neurotoxins using C6 glioma cells

The culture medium was first removed from each well and C6 cells were washed twice with warm (37 °C) Hank's solution. The cells were then incubated separately for 10 min in the presence and absence of paralytic shellfish poisons (e.g., GTX2,3 and GTX1,4) with 270 μL Hank's solution (pH 7.4) supplemented with POD (64 U/mL), luminol (0.05 mM) and L-glutamate oxidase (17 U/mL) prior to stimulation. The cells were stimulated by auto-injection of 30 μL solution of the activator, veratridine, NMDA or ibotenic acid using a luminescence plate-reader (FLUOstar OPTIMA, BMG LABTECH, Germany). Hank's solution (pH 7.4, 30 μL) was injected as a stimulant for the control experiments.

2.5. Assay of the release of L-glutamate in real-time from rat cortical neurons

The culture medium was first removed from each well and the cortical neurons were washed twice with warm (37 °C) Hank's solution. The cells were then incubated for 20 min with 270 μL measurement solution (205 μL Hank's solution, 30 μL POD, 30 μL luminol and 5 μL L-glutamate oxidase) prior to stimulation. The cells were stimulated by injection of 30 μL solution of either K^+ or NMDA using a luminescence plate-reader. Hank's solution was injected as a stimulant for the control experiments.

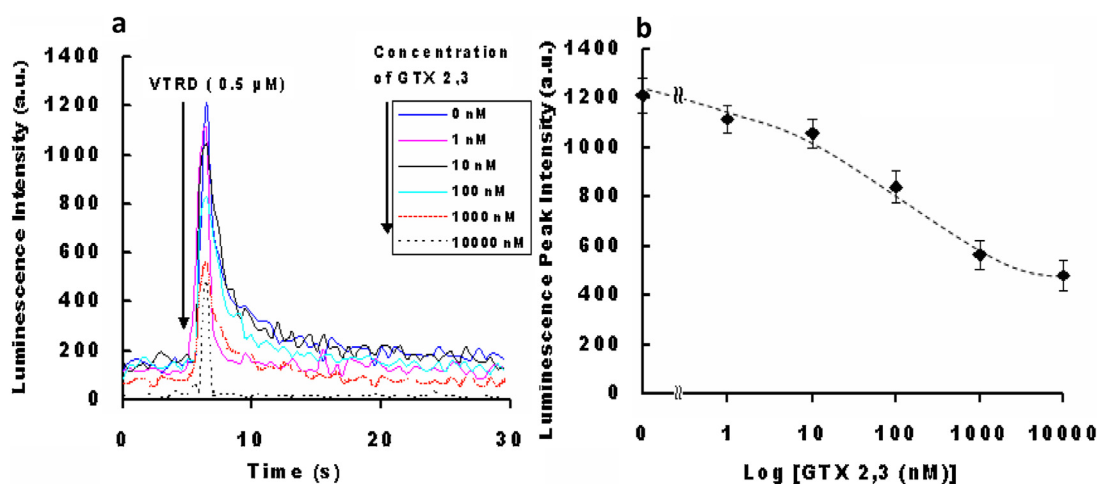


Fig. 2. Effects of GTX2,3 on the L-glutamate response upon stimulation by veratridine (0.5 μM). C6 glioma cells were incubated for 10 min prior to stimulation in the presence and the absence of GTX2,3. (a) Time courses of luminescence intensity monitoring of L-glutamate release from C6 cells in the presence and absence of GTX2,3 at several concentrations (0–10 μM). (b) Dose-response inhibitory effects of GTX2,3 on the L-glutamate response to veratridine (0.5 μM) at the indicated concentrations in (a). All points represent the mean ± SD of six experiments at each concentration.

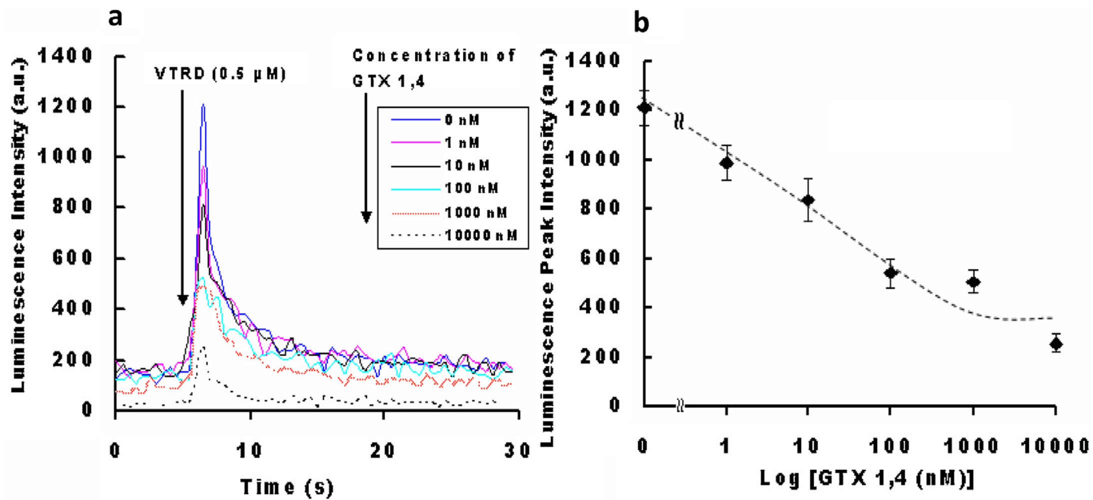


Fig. 3. Effects of GTX1,4 on the L-glutamate response to veratridine (0.5 μM). C6 glioma cells were incubated for 10 min in the presence and absence of GTX1,4 prior to stimulation. (a) Time courses of luminescence intensity indicating L-glutamate release from C6 cells in the presence and absence of GTX1,4 at several concentrations (0–10 μM). (b) Dose-dependent inhibitory effects of GTX1,4 on the release of L-glutamate to veratridine (0.5 μM) at the indicated concentrations in (a). Data are mean ± SD of six determinations at each concentration.

2.6. Assay of glutamate residues from rat cortical neurons

After the assay of glutamate release, the solution was replaced with 300 μL Hank's solution and the cells were disrupted by either sonication (power 7, 40 s) or freezing (−20 °C, overnight). The sample was separated following centrifugation (13.2×10^3 rpm, 6 min) by filtration using a membrane filter (pore size 0.22 μM). For recoding the L-glutamate residue, the measurement solution supplemented with Hank's buffer (pH 7.4, 205 μL), 64 U/mL POD (30 μL), 0.05 mM luminol (30 μL), and 17 U/mL L-glutamate oxidase (5 μL) was added to each well of a 24-well plate. Purified sample solution (30 μL) was injected into the wells of the 24-well

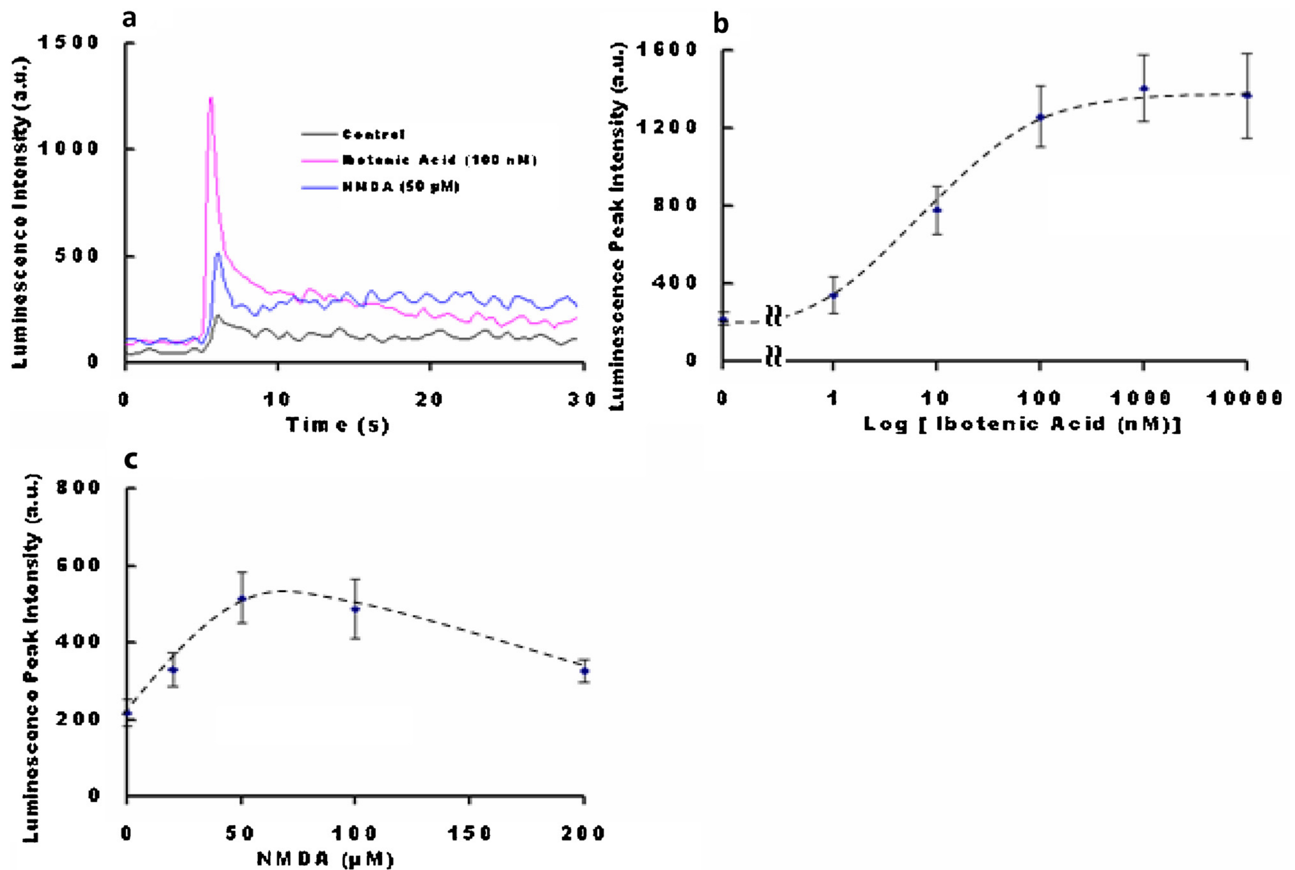


Fig. 4. Effects of NMDA receptor agonists including mushroom toxin and NMDA on the L-glutamate responses from C6 glioma cells. (a) Time courses of luminescence intensity indicating L-glutamate release from C6 cells upon stimulations by ibotenic acid, and NMDA at the indicated concentrations. Dose-dependent effects of (b) ibotenic acid (0–10 μM), and (c) NMDA (0- 200 μM) activators on the release of L-glutamate from the cells. Hank's solution was used as a stimulant for the control experiments. Data are mean ± SD of six determinations at each concentration.

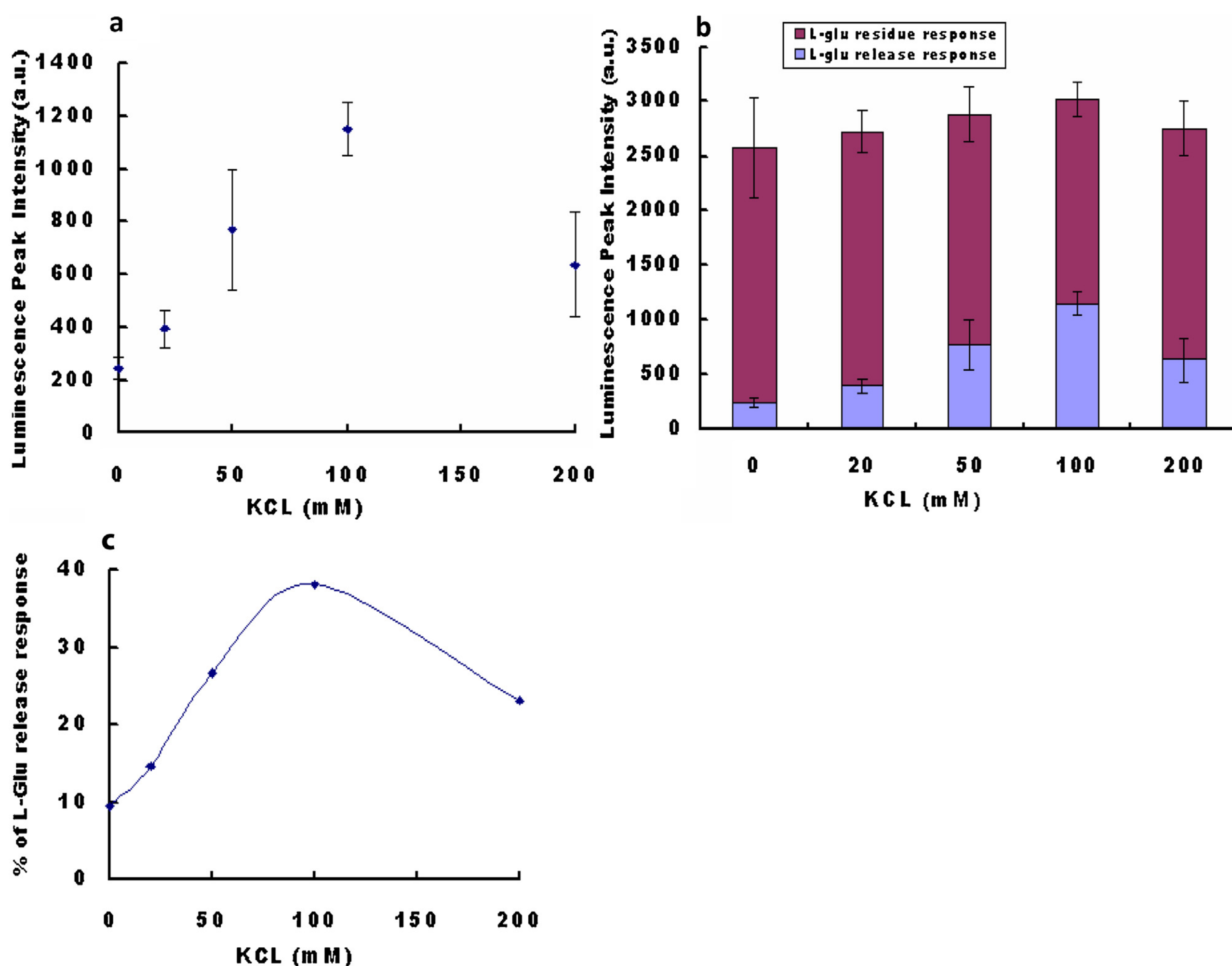


Fig. 5. Real-time monitoring of L-glutamate release from primary cortical neurons. (a) Dose-dependent effects of KCl at several concentrations (0–200 mM) on the release of L-glutamate from cortical neurons. (b) Released and unreleased glutamate from neurons due to the effects of KCl at each concentration in (a). (c) Percentage release of glutamate due to the effects of KCl at each concentration in (a). All points are mean ± SD of four measurements at each concentration.

plate using an automatic reagent injector to start the enzymatic reaction and luminescence was detected using a luminescence plate-reader equipped with a highly sensitive photomultiplier tube.

3. Results and discussion

3.1. Effects of paralytic shellfish toxins on veratridine-induced glutamate release from C6 cells

In order to investigate the effects of paralytic shellfish toxins, the cells were pretreated separately with racemic mixtures of the gonyautoxins, including GTX2,3 and GTX1,4, for 10 min prior to stimulation with veratridine (final conc., 0.5 μM), and the glutamate release from the cells was monitored in real-time. Fig. 2a shows time courses of L-glutamate response measured by the enzyme-luminescence method in the presence and absence of GTX2,3 at the indicated concentrations. Fig. 2b shows a semi-logarithmic plot of the luminescence peak intensity corresponding to veratridine-evoked L-glutamate release in the same experiments. Similarly, Fig. 3a and b shows the time courses of L-glutamate response and a semi-logarithmic plot of the luminescence peak intensity, respectively, in the presence and absence of GTX1,4 at the indicated concentrations. The results indicate that increasing concentrations of both GTX2,3 and GTX1,4 progressively inhibit the veratridine-induced release of glutamate. Several studies have suggested that gonyautoxins block $Na_v1.4$ channel, which may decrease or delay neuronal depolarization as well as glutamate release [17,18,23,31] The data reported here support this phenomena.

The calculated IC_{50} values for GTX2,3 and GTX1,4 to inhibit veratridine-induced glutamate release are 30 nM and 8 nM, respectively, which are higher than those reported by Choudhary et al. [23]. This discrepancy is probably due to the difference of both the method of analysis and the cells used. However, the results are consistent with the data reported previously [23] showing that GTX1,4 is the more potent paralytic shellfish toxin for blocking voltage-gated Na^+ channels as compared to GTX2,3.

3.2. Effects of ibotenic acid on the release of L-glutamate from C6 cells

Ibotenic acid, isolated from mushrooms, is a powerful natural neurotoxin that can bind NMDA receptors and keep them open [1,2–4]. The activation of NMDA receptors promote the influx of cations (e.g., Na^+, Ca^{2+}), which can lead to membrane depolarization. This, in turn, can help trigger exocytosis of neurotransmitters such as L-glutamate [1,2,3,10,12]. The C6 glioma cells were stimulated by ibotenic acid to evaluate the effects of the natural toxin on NMDA-receptor-mediated L-glutamate release. The potency of this mushroom toxin was compared with that of

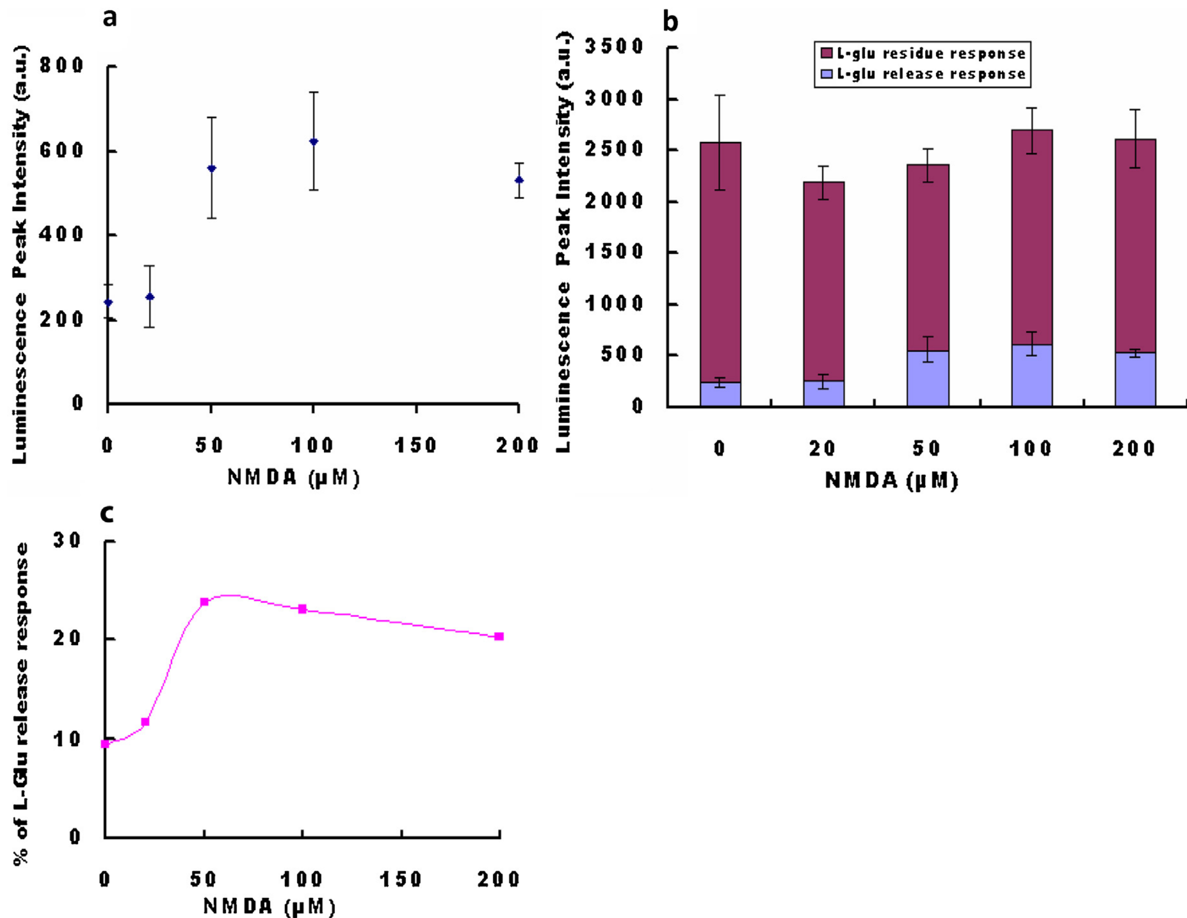


Fig. 6. Real-time monitoring of L-glutamate release from primary cortical neurons. (a) Dose-dependent effects of NMDA at several concentrations (0–200 μM) on the release of L-glutamate from the neurons. (b) Released and unreleased glutamate from neurons due to the effects of NMDA at each concentration in (a). (c) Percentage release of glutamate due to the effects of NMDA at each concentration in (a). All points are mean ± SD of four measurements at each concentration.

NMDA, a specific well-known agonist of the NMDA receptor. Fig. 4a shows the time courses of L-glutamate release evoked by ibotenic acid and NMDA at the indicated concentrations. The luminescence peak intensities are significantly higher for both cases than the control, which is consistent with that of the previous reports [1–3,10,12].

The higher luminescence peak intensity (peak height) is most probably due to either the increase of H_2O_2 produced by the L-glutamate efflux upon stimulation or the oxidation of stimulants (e.g., ibotenic acid, NMDA) themselves by L-glutamate oxidase. The stimulation experiment was conducted using either ibotenic acid or NMDA in the absence of cells to confirm the specificity. The

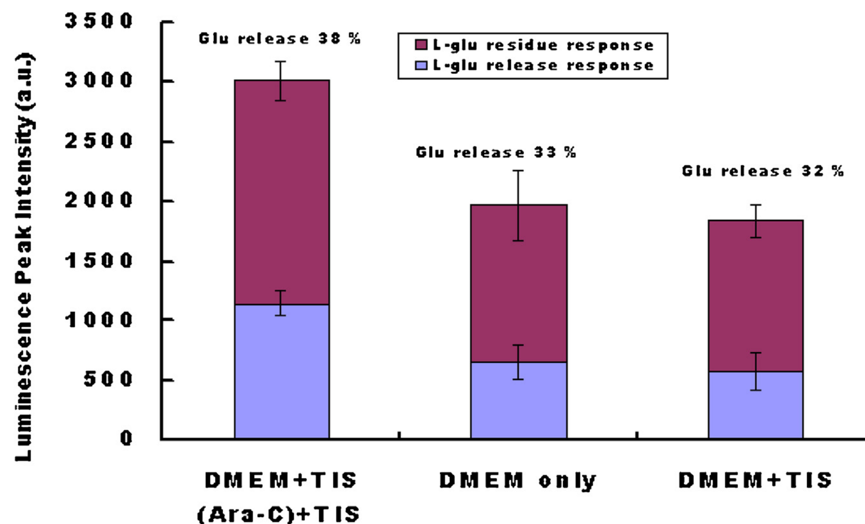


Fig. 7. Effects of different culture conditions on the L-glutamate response to KCl. (100 mM). Percentage release of glutamate due to the effects of different culture media is also shown. All points are mean ± SD of four measurements at each concentration.

results (not shown) are similar to those of the control experiments, indicating that ibotenic acid and NMDA are not oxidized by L-glutamate oxidase. Therefore, the observed high luminescence intensity is due to the release of L-glutamate from the cells.

Fig. 4b shows a semi-logarithmic plot of the dose-dependence of ibotenic acid on the release of L-glutamate from C6 cells, in which the luminescence peak intensity (peak height) increases with increasing ibotenic acid concentration. The data also indicate that the minimum level of ibotenic acid that evokes a distinguishable luminescence response from cells over the control is 1 nM. Similarly, Fig. 4c shows the dependence of the luminescence peak intensity (peak height) on concentration upon stimulation with NMDA (0–200 μM). Saturated level is 50 μM NMDA and a much higher concentration of glutamate is released with this dose as compared with the control. Furthermore, the EC_{50} values for ibotenic acid- and NMDA-induced glutamate release were calculated to be 10 nM and 22 μM, respectively. The results demonstrate that ibotenic acid is a more potent stimulant for the activation of NMDA receptors than NMDA. Moreover, the binding affinity of ibotenic acid to NMDA receptors is higher than that of NMDA. The data are consistent with the results reported previously [2,12].

3.3. Real-time monitoring of L-glutamate release from primary cortical neurons

The effects of two types of stimulants, such as KCl and NMDA on L-glutamate release from primary cortical neurons were also investigated. The luminescence peak appeared rapidly, within a few seconds, after the injection of either KCl or NMDA solution. Fig. 5a indicates that the luminescence peak intensity depends on the concentration of KCl. The data indicate that the luminescence intensity reaches a maximum at about 100 mM KCl, which is consistent with the observations in previous reports [27,32–34] Residue of glutamate was also measured after breaking the cells following centrifugation and filtration. The total amount of glutamate (released + unreleased) from cortical neurons determined in terms of luminescence peak intensities is shown in Fig. 5b. Fig. 5c shows the percentage of glutamate released as a function of the concentration of KCl, where the maximum glutamate release was observed at 100 mM KCl.

Fig. 6a shows the dependence of the luminescence peak intensity on concentration upon stimulation with NMDA, where the peak intensity reaches a maximum at an NMDA concentration of 100 μM. Fig. 6b shows a bar chart indicating the total glutamate response. Fig. 6c shows the percentage of glutamate released as a function of the concentration of NMDA, where the maximum amount of glutamate released was observed at 50–100 μM NMDA.

3.4. Effects of different culture conditions on glutamate release due to depolarization induced by K^+

The primary cortical neurons were grown separately under different culture conditions, (i.e., DMEM + TIS with Ara-C + TIS, DMEM only, and DMEM + TIS), and the neurons were stimulated with a high concentration of KCl (100 mM) to monitor the glutamate released from the neurons. Fig. 7 shows the peak intensities due to the released and residual glutamate at different culture conditions. The percentage of glutamate released in the presence of all three media, two media, and one medium were calculated to be 38%, 32%, and 33%, respectively. The total amount of glutamate released in the presence of all three media is high compared to that of the other two culture conditions. This is probably due to the high rate of differentiation from primary neurons into glutamatergic neurons when three media are used sequentially. The increased glutamate released upon stimulation with an agonist may be a good marker of differentiation in primary cortical neurons, which is truly a dye free marker. A majority of conventional techniques used to monitor neuronal differentiation in vitro are based on immunostaining and are dependent on a fluorescent or radioactive dye [35–37]. Thus, the enzyme-luminescence method may be a useful tool in the evaluation of neuronal differentiation ratio non-invasively, in real-time and independent of any fluorescent or radioactive probes.

4. Conclusion

The applicability of the enzyme-luminescence method reported earlier for in vitro screening of natural neurotoxins is demonstrated. Racemic mixtures of the gonyautoxins, containing GTX2,3 and GTX1,4, were used to evaluate their inhibition of the release of glutamate from the C6 glioma cells. The activation of the release of glutamate from C6 cells by ibotenic acid (a mushroom toxin) was also evaluated. In order to compare the potency of these toxins, EC_{50} or IC_{50} values were calculated. Furthermore, the enzyme-luminescence method is useful for the in vitro detection of the release of L-glutamate from primary cortical neurons. This novel detection technique may also be used to determine the neuronal differentiation ratio and to find glutamatergic neurons in real-time without immunostaining. Thus, the cell-response-based sensing technology may find widespread applications in screening of other drugs and toxins that activate or inhibit either ion-channels or receptors. This sensing tool may also have great potential for the investigation of the effects of various growth factors and chemicals on neuronal differentiation, neurotransmitter dynamics, neurodegeneration, and synaptogenesis.

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References

[1] G. Herrmann, H. Stünitz, C. Nitsch, Composition of ibotenic acid-induced calcifications in rat substantia nigra, Brain Res. 786 (2010) 205–214.
[2] M.B. Hermit, J.R. Greenwood, B. Nielsen, L. Bunch, C.G. Jørgensen, H.T. Vestergaard, T.B. Stensbøl, C. Sanchez, P. Krogsgaard-Larsen, U. Madsen, H. Bräuner-Osborne, Ibotenic acid and thioibotenic acid: a remarkable difference in activity at group III metabotropic glutamate receptors, Eur. J. Pharmacol. 486 (2004) 241–250.
[3] C.G. Jørgensen, H. Bräuner-Osborne, B. Nielsen, J. Kehler, R.P. Clausen, P. Krogsgaard-Larsen, U. Madsen, Novel 5-substituted 1-pyrazolol analogues of ibotenic acid: synthesis and pharmacology at glutamate receptors, Bioorg. Med. Chem. 15 (2007) 3524–3538.
[4] T.V.P. Bliss, G.L. Collingridge, A synaptic model of memory: long-term potentiation in the hippocampus, Nature 361 (1993) 31–39.
[5] S.F. Traynelis, L.P. Wollmuth, C.J. McBain, F.S. Menniti, K.M. Vance, K.K. Ogden, K.B. Hansen, H. Yuan, S.J. Myers, R. Dingledine, Glutamate receptor ion channels: structure, regulation, and function, Pharmacol. Rev. 62 (3) (2010) 405–496.
[6] S. Nakanishi, Molecular diversity of glutamate receptors and implications for brain function, Science 258 (1992) 597–603.
[7] L. Kergoat, B. Piro, D.T. Simon, M.C. Pham, V. Noël, M. Berggren, Detection of glutamate and acetylcholine with organic electrochemical transistors based on conducting polymer/platinum nanoparticle composites, Adv. Mater. 26 (32) (2014) 5658–5664.
[8] M.R. Hynd, H.L. Scott, P.R. Dodd, Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease, Neurochem. Int. 45 (5) (2004) 583–595.

[9] A. Takeda, A. Minami, N. Sakurada, S. Nakajima, O. Naoto, Response of hippocampal mossy fiber zinc to excessive glutamate release, Neurochem. Int. 50 (2) (2007) 322–327.
[10] P. Sun, S.G. Rane, P.G. Gunasekar, J.L. Borowitz, G.E. Isom, Modulation of the NMDA receptor by cyanide: enhancement of receptor-mediated responses, Pharmacol. Exp. Ther. 280 (3) (1997) 1341–1349.
[11] D.T. Monaghan, M.W. Irvine, B.M. Costa, G. Fang, D.E. Jane, Pharmacological modulation of NMDA receptor activity and the advent of negative and positive allosteric modulators, Neurochem. Int. 6 (4) (2012) 581–592.
[12] J. Arnt, C. Sánchez, S.M. Lenz, U. Madsen, P. Krogsgaard-Larsen, Differentiation of in vivo effects of AMPA and NMDA receptor ligands using drug discrimination methods and convulsant/anticonvulsant activity, Eur. J. Pharmacol. 285 (1995) 289–297.
[13] R.C.C. Cianca, M.A. Pallares, R.D. Barbosa, L.V. Adan, J.M.L. Martins, A. Gago-Martínez, Application of precolumn oxidation HPLC method with fluorescence detection to evaluate saxitoxin levels in discrete brain regions of rats, Toxicon 49 (1) (2007) 89–99.
[14] M. Janecek, M.A. Quilliam, J.F. Lawrence, Analysis of paralytic shellfish poisoning toxins by automated pre-column oxidation and microcolumn liquid chromatography with fluorescence detection, J. Chromatogr. 644 (1993) 321–331.
[15] W.M. Indrasena, R.G. Ackman, T.A. Gill, Separation of paralytic shellfish poisoning toxins on Chromarods-SIII by thin-layer chromatography with the Iatroscan (mark 5) and flame thermionic detection, J. Chromatogr. A 855 (2) (1999) 657–668.
[16] W.M. Indrasena, T.A. Gill, Fluorometric detection of paralytic shellfish poisoning toxins, Anal. Biochem. 264 (2) (1998) 230–236.
[17] M. Okumura, H. Tsuzuki, B. Tomita, A rapid detection method for paralytic shellfish poisoning toxins by cell bioassay, Toxicon 46 (1) (2005) 93–98.
[18] R. Manger, D. Woodle, A. Berger, J. Hungerford, Flow cytometric detection of saxitoxins using fluorescent voltage-sensitive dyes, Anal. Biochem. 366 (2007) 149–155.
[19] D.S. Kerr, D.M. Briggs, H.I. Saba, A neurophysiological method of rapid detection and analysis of marine algal toxins, Toxicon 37 (12) (1999) 1803–1825.
[20] P. Ginterová, B. Sokolová, P. Ondra, J. Znaleziona, J. Petr, J. Ševčík, V. Maier, Determination of mushroom toxins ibotenic acid: muscimol and muscarine by capillary electrophoresis coupled with electrospray tandem mass spectrometry, Talanta 125 (2014) 242–247.
[21] A. Poliwoda, K. Zielińska, M. Halama, P.P. Wieczorek, Determination of muscimol and ibotenic acid in mushrooms of Amanitaceae by capillary electrophoresis, Electrophoresis 35 (18) (2014) 2593–2599.
[22] R.Y. Shimojo, W.T. Iwaoka, A rapid hemolysis assay for the detection of sodium channel-specific marine toxins, Toxicology 154 (1–3) (2000) 1–7.
[23] G. Choudhary, L. Shang, X. Li, S.C. Dudley Jr., Energetic localization of *saxitoxin* in its channel binding site, Biophys. J. 83 (2002) 912–919.
[24] S.J. Locke, P. Thibault, Improvement in detection limits for the determination of paralytic shellfish poisoning toxins in shellfish tissues using capillary electrophoresis/electrospray mass spectrometry and discontinuous buffer systems, Anal. Chem. 66 (1994) 3436–3446.
[25] J.C. Oliveira, S.C.R. Fernandes, C.A. Schwartz, C. Bloch Jr., J.A.T. Melo, O.R. Pires Jr, J.C. De Freitas, Toxicity and toxin identification in *Colomesus asellus*, an Amazonian (Brazil) freshwater puffer fish, Toxicon 48 (1) (2006) 55–63.
[26] F.G. Camacho, J.G. Rodríguez, A.S. Mirón, M.C.C. García, E.H. Belarbi, Y. Chisti, E. M. Grima, Biotechnological significance of toxic marine dinoflagellates, Biotechnol. Adv. 25 (2) (2007) 176–194.
[27] S.M.Z. Hossain, H. Shinohara, F. Wang, H. Kitano, Real-time detection of L-glutamate released from C6 glioma cells using a modified enzyme-luminescence method, Anal. Bioanal. Chem. 389 (6) (2007) 1961–1966.
[28] S.M.Z. Hossain, H. Shinohara, H. Kitano, Drug assessment based on detection of L-glutamate released from C6 glioma cells using an enzyme-luminescence method, Anal. Chem. 80 (2008) 3762–3768.
[29] H. Shinohara, F. Wamg, S.M.Z. Hossain, A convenient, high-throughput method for enzyme-luminescence detection of dopamine released from PC12 cells, Nat. Protoc. 3 (10) (2008) 1639–1644.
[30] T.A. Mir, H. Shinohara, Y. Shimizu, Enzyme-luminescence method: Tool for evaluation of neuronal differentiation based on real-time monitoring of dopamine release response from PC12 cells, Anal. Methods 3 (2011) 837–841.
[31] C.P. Taylor, B.S. Meldrum, Na^+ channels as targets for neuroprotective drugs, Trends Pharmacol. Sci. 16 (9) (1995) 309–316.
[32] S. Isik, J. Castillo, A. Blöchl, E. Csöregi, W. Schuhmann, Simultaneous detection of L-glutamate and nitric oxide from adherently growing cells at known distance using disk shaped dual electrodes, Bioelectrochemistry 70 (1) (2007) 173–179.
[33] A.M. Villagran, R. Tapia, Preferential stimulation of glutamate release by 4-aminopyridine in rat striatum in vivo, Neurochem. Int. 28 (1) (1996) 35–40.
[34] J. Castillo, S. Isik, A. Blöchl, N. Pereira-Rodrigues, F. Bedioui, E. Csöregi, W. Schuhmann, J. Oni, Simultaneous detection of the release of glutamate and nitric oxide from adherently growing cells using an array of glutamate and nitric oxide selective electrodes, Biosens. Bioelectron. 20 (8) (2005) 1559–1565.
[35] G. Daoud, E. Rassart, A. Masse, J. Lafond, Src family kinases play multiple roles in differentiation of trophoblasts from human term placenta, J. Physiol. 571 (3) (2006) 537–553.
[36] Y. Merot, F. Ferriere, E. Debroas, G. Flouriot, Estrogen receptor alpha mediates neuronal differentiation and neuroprotection in PC12 cells: critical role of the A/B domain of the receptor, J. Mol. Endocrinol. 35 (2005) 257–267.
[37] T.E. Jin, M. Jang, H. Kim, Y.M. Choi, H. Cho, S. Chung, M.K. Park, Involvement of corticotropin-releasing factor receptor 2 beta in differentiation of dopaminergic MN9D cells, Mol. Cells 26 (3) (2008) 243–249.